B3

(a) providing an animal cell transfected with a nucleic acid molecule that contains (i) a nucleic acid sequence encoding a biofilament, (ii) a promoter that directs expression of a polypeptide in an animal cell, wherein said promoter is operably linked to said nucleic acid sequence encoding said biofilament, and (iii) a leader sequence that causes secretion of said biofilament by said cell;

(b) culturing said transfected cell under conditions in which said biofilament is secreted into the culture medium of said cultured cell; and

(c) isolating said biofilament from [the] said culture medium of said cultured transfected cell.

REMARKS

The invention as now claimed provides nucleic acid molecules comprising a nucleic acid sequence encoding a biofilament; a promoter operably linked to the nucleic acid sequence that directs expression of a polypeptide in milk-producing or urine-producing cells; and a leader sequence that enables secretion of the biofilament by the milk-producing or urine-producing cells into milk or urine, respectively, of a mammal. The invention also features transgenic animals containing the above-described nucleic acid molecule that secrete the biofilament. In addition, the present invention features a transgenic embryo containing the biofilament-encoding nucleic acid construct, which may be used to generate the adult animals from which the biofilament is produced. Finally, the invention provides methods of producing biofilaments from a transgenic animal or from cultured cells.

Claims 1-21 were examined in this case. Claims 2, 3, and 5 were rejected under 35 U.S.C. § 101, claims 1-21 were rejected under 35 U.S.C. § 112, first paragraph, and claims 2-6 and 13-21 were rejected under 35 U.S.C. § 112, second paragraph. Each of the rejections raised in the Office Action are addressed individually below.

Rejections Under 35 U.S.C. 101

Claims 2, 3, and 5 were rejected under 35 U.S.C. § 101, with the Examiner stating that the claims, drawn to a transgenic mammalian embryo or animal, encompass a human embryo or humans, which are non-statutory subject matter. The Examiner suggested that insertion of the phrase "non-human" into the rejected claims would overcome this rejection. Such claim amendments have been made and this rejection can now be withdrawn.

Claim 2 was also rejected under 35 U.S.C. § 101, based on the assertion by the Office that the claimed invention is not supported by either a specific asserted utility or a well-established utility. Applicants respectfully disagree.

Claim 2, as amended, is drawn to a non-human mammalian embryo comprising a cell whose nucleus comprises the nucleic acid molecule of claim 1. Applicants assert that the embryo of claim 2 has a specific utility. The embryo of claim 2 is used to generate the adult animal from which the desired biofilaments of the invention are obtained. For example, the specification, at pages 26-27, states that embryonic stem cells may be transformed with a transgene. These transformed embryonic stem cells are then combined with blastocysts from the animal from which they originate. The cells colonize the embryo, and in some embryos these cells form the germline of the resulting chimeric animal. These chimeric animals are then bred to generate the transgenic animals that secrete biofilaments. Applicants assert that the utility of the embryos of claim 2 has been established, and withdrawal of this rejection is requested.

Rejections Under 35 U.S.C. § 112, first paragraph

Claim 2 was rejected under 35 U.S.C. § 112, first paragraph on the ground that since the claimed invention is not supported by either a specific asserted utility or a well-established utility, one skilled in the art would clearly not know how to use the claimed

invention.

In response, Applicants submit that the rejection of claim 2 under 35 U.S.C. § 101 has been overcome. As the utility of the embryo of claim 2 has been established, Applicants submit that one skilled in the art would know that the embryo of claim 2 may be used to generate an adult animal from which secreted biofilaments may be obtained. Applicants respectfully request withdrawal of this rejection.

Claims 3-6 were rejected under 35 U.S.C. § 112, first paragraph, based on the assertion by the Examiner that the claims do not recite any particular phenotype for the claimed transgenic animals. The Examiner suggested that this rejection could be overcome by amending the claims to recite the phenotype of the claimed animals. Such amendments have been made. Support for the amendments is found in the specification at pages 26-29. Accordingly, this portion of the rejection may now be withdrawn.

Claims 1 and 3-21 were rejected under 35 U.S.C. § 112, first paragraph, with the Examiner stating that the specification does not offer adequate guidance to teach one skilled in the art how to produce any transgenic animal that expresses a biofilament in milk or urine to a level sufficient to allow purification of the biofilament from the biological fluid. The Examiner also argues that the specification fails to provide an enabling disclosure because the recited nucleic acid constructs are not enabled for their intended use. Applicants respectfully disagree.

The standard for enablement is articulated in *In re Wands* 858 F 2d. 731, 8 USPQ2d 140, 1402 (Fed. Cir. 1988). *Wands* sets forth eight factors to be taken into account to determine whether the experimentation necessary to practice the scope of the claimed invention is "undue." These factors are addressed below.

Nature of the Invention: As they relate to this portion of the § 112, first paragraph rejection, the claimed invention features nucleic acid molecules comprising a nucleic acid sequence encoding a biofilament; a promoter operably linked to the nucleic acid sequence

that directs expression of a polypeptide in milk-producing cells or urine-producing cells; and a leader sequence that enables secretion of the biofilament by the milk-producing or urine-producing cells into milk or urine, respectively, of a mammal. The claimed invention also features transgenic animals containing the above-described nucleic acid molecules that secrete the biofilament. Finally, the claimed invention provides methods of producing biofilaments from a transgenic animal.

Applicants submit that, given the teaching of the specification and the level of skill known in the art at the time the present application was filed, as detailed below, one skilled in the art could have readily generated the nucleic acid molecules and biofilament-producing transgenic animals of the present invention, without undue experimentation, and of the full scope of the claims.

Amount of Guidance Provided in the Specification: The specification provides ample guidance for claims of the present scope. For example, the specification, at pages 9-19, provides a detailed description of a large number of silk genes, urine- or milk-specific promoters, signal sequences, and termination regions that may be used to construct the nucleic acid molecules of the present invention. The specification, at pages 21-24, also provides specific constructs that may be used to practice the present invention. For example, pages 21-24 describe a construct comprising the goat β-casein promoter, followed by its own signal sequence for expression, followed by a 1.5 kb insert containing the silk clone in frame with the 5' and 3' ends of the casein gene. A schematic diagram of this construct is shown in Fig. 1A of the application. In another example, the specification teaches a nucleic acid construct comprising the WAP gene promoter, its signal sequence, a 1.5 kb cDNA encoding dragline silk, followed by the 3' end of the WAP gene. The details of its construction are diagramed in Fig. 1B of the application.

In yet another example of a nucleic acid molecule provided in the specification, the construct comprises the uroplakin II promoter, which is used to drive expression of

the fibroin or silk gene(s) in the urothelium of transgenic animals. The fibroin or silk gene(s) is inserted downstream of a 3.6 kb 5' flanking sequence of the mouse uroplakin II (UPII) gene. A sequence containing part of exon 1 and all of intron 1 and exon 2 of the mouse protamine-1 (Mp-1) gene is placed at the 3' end of the gene to provide an exon/intron splicing site and a polyadenylation signal. A diagram of this construct is shown in Fig. 1C of the application.

The specification also describes a protocol that is generally applicable for generating transgenic animals that express a biofilament in their milk or urine. At pages 26-29, the specification teaches that transgenes may be introduced into the pronuclei of fertilized oocytes, for example, by microinjection. The microinjected zygotes are transferred to an appropriate female, resulting in the birth of a transgenic or chimeric animal, depending upon the stage of development when the transgene is integrated. Chimeric animals can be bred to form true germline transgenic animals.

The specification also teaches another method for the generation of transgenic animals that secrete biofilaments, by introducing the biofilament-encoding transgene into embryonic stem cells (ES cells). In particular, the specification states that transgenes can be introduced into such cells by electroporation, microinjection, or any other techniques used for the transfection of cells which are known to the skilled artisan. Transformed cells are combined with blastocysts from the animal from which they originate. The cells colonize the embryo, and in some embryos these cells form the germline of the resulting chimeric animal. Alternatively, ES cells can be used as a source of nucleic for transplantation into an enucleated fertilized oocytes, thus giving rise to a transgenic animal.

Level of Skill in the Art: The level of skill in the pertinent art is very high; most practitioners hold Ph.D. degrees, and many have significant post-doctoral experience. Applicants submit that one skilled in the fields of molecular biology and genetics could

have readily designed a great many of the nucleic acid constructs of the invention as presently claimed without undue experimentation, and could have successfully generated a wide range of transgenic animals using the specification for guidance. The Examiner has not presented convincing evidence or arguments to the contrary.

Furthermore, one of ordinary skill in the art would immediately recognize that the teachings of the specification are easily transferred to other nucleic acid constructs and animals. Moreover, the law does not require that every embodiment encompassed by a claim be operable. Thus, even if a procedure described in the specification would not achieve production of a biofilament in every conceivable animal, this would not negate enablement of the present claims.

Working Examples: Applicants provide a fully enabling disclosure of how to practice the claimed invention. While they do not present working examples, they note that none is required. Applicants point out that the Federal Circuit has made clear the level of teaching needed to enable a claim with respect to the number of working examples, and has stated that a specification need not contain a working example if the invention is otherwise disclosed in such a manner that one skilled in the art is able to practice it without undue experimentation. See *In re Walter L. Borkowski and John J. Van Venrooy*, 422 F2d 904, 164 UPSQ 642 (Fed. Cir. 1970) (eleven step method for preparing an oxygenated hydrocarbon, found to be enabled by the specification absent a working example). See also *In re Roger A. Long*, 368 F.2d 892, 151 USPQ 640 (Fed. Cir. 1966). ("The absence of a working example, denominated as such, does not compel the conclusion that a specification does not satisfy the requirement of 35 USC 112...").

State of the Prior Art: Nucleic acid sequences encoding biofilaments, as disclosed in the present specification, were known in the art at the time the present application was filed (see, for example, Arcidiacono et al., Appl. Microbiol. Biotechnol. 49:31-38, 1998; previously filed in an Information Disclosure Statement for this case).

Promoters specific for milk-producing cells or urine-producing cells were also known prior to the filing date of the present application. For example, prior to Applicants' filing date, promoters for the expression of a polypeptide in milk were known to include: murine WAP (Velander et al., Proc. Natl. Acad. Sci USA 89:12003-12007, 1992); bovine αS1-casein (PCT Application Nos.: WO 91/08216 and WO 93/25567); γ-casein and rat β-casein (Rosen, U.S. Patent No. 5,304,489); sheep β-lactoglobulin (Wright et al., Bio/Technology 9:830-834, 1991; Exhibit A); goat β-casein (Ebert et al., Bio/Technology 12:699-702, 1994); and bovine α-lactalbumin (Vilotte et al., Eur. J. Biochem. 186:43-48, 1989). Promoters for expression of polypeptides in the urine were also known in the prior art. For example, Kerr et al. discloses the use of the murine bladder-specific uroplakin II promoter to express recombinant human growth hormone, while Sun describes the use of the same promoter to express β-galactosidase (Nature Biotechnology 16:75-79, 1998; and Sun, WO 96/39494). Applicants point out that copies of Arcidiacono et al., Velander et al., PCT Application Nos.: WO 91/08216 and WO 93/25567, U.S. Patent No. 5,304,489, Ebert et al., Vilotte et al., Kerr et al., and WO 96/39494 were previously submitted in Information Disclosure Statements. As the Office should have copies of these references, Applicants have not submitted them as Exhibits.

Methods for the generation of transgenic animals that produce desired polypeptides that are secreted into the urine or milk of the animal were known for a variety of animals prior to the filing date of the present application. For example, Velander et al. generated transgenic swine and mice that produce human protein C in their milk (supra; and Annals New York Academy of Sciences 665:391-403, 1992; Exhibit B); Wright et al. (supra) generated transgenic sheep that produce human α-1-antitrypsin in their milk; Ebert et al. (supra) produced transgenic goats that produce human longer acting tissue plasminogen activator in their milk; and Kerr et al. (supra) produced transgenic mice that produce human growth hormone in their urine.

Furthermore, methods for purifying polypeptides from the milk or urine of animals was known prior to Applicants' filing date. For example, Deboer et al. (WO 93/25567) describes the isolation of heterologous proteins from the milk of bovine species. In addition, Applicants note that methods for producing silk fibers from aqueous solutions of silk polypeptides from silkworms were known in the art prior to Applicants' filing date. For example, Lock et al. teach methods for spinning silk fibroin polypeptides into silk fibers (U.S. Patent No. 5,252,285; Exhibit C).

Quantity of Experimentation Necessary: The Examiner states on page 5 of the Office Action that, in view of the lack of working examples in the specification and the unpredictability in the art, one of ordinary skill in the art would have been required to engage in undue experimentation in order to make and use the transgenic animals of claims 3-6. The Examiner further states, at page 7 of the Office Action, that undue experimentation would have been required for one skilled in the art to produce a biofilament, as recited in claims 13-21, in any cell, particularly a milk-producing cell or a urine-producing cell.

Applicants submit that the Examiner has reinterpreted the *Wands* factors to render them more stringent than the statute or case law, including *Wands*, permits. Under the standard of enablement, an Applicant is required to provide sufficient information to allow practice of the invention, not to prove that <u>all</u> possible embodiments will work.

In re Wands involved a method for identifying monoclonal antibodies that are specific for a particular antigen. The method required screening large numbers of hybridomas to determine which ones secrete an antibody with the desired characteristics. There was no question but that identification of useful hybridoma lines required substantial experimentation, and was a rare event. The broad claim was held enabled nonetheless. Similarly, the present invention may require testing, based on a variety of predefined parameters, in order to optimize the method for making transgenic animals

and biofilaments described in the specification. This does not mean the claims (as amended) are not enabled.

The specification teaches that the described methods of generating a transgenic animal that secretes a biofilament, and for producing biofilaments from transgenic animals, can be repeated in any species, and provides ample guidance to allow the skilled artisan to carry this out, with routine experimentation. As was stated *In re Wands*, "a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." As the specification teaches nucleic acid constructs and methods for their use in the generation of transgenic animals that secrete biofilaments (see, for example, pages 21-29), the experimentation necessary to practice the invention as presently claimed is clearly not undue.

Predictability: The Examiner argues that in the absence of specific guidance, the existence of any phenotypic alteration resulting from the introduction of a nucleic acid construct comprising a biofilament operably linked to a milk-specific or urine-specific promoter in any species of animal is highly unpredictable. The Examiner cites a reference, Wall et al. (Theriogenology 45:57-68, 1996), to show that insertional inactivation of endogenous genes and positional effects can influence the phenotype of the resultant transgenic animal, that expression of the transgene and its effect on the phenotype of the transgenic animal are unpredictable, and that the genetic elements required for appropriate expression vary from species to species.

Applicants submit that the information provided by the Wall et al. reference actually demonstrates that the state of the transgenic art at the time of filing was such that transgene expression was predictable among various species of mammals. Table 1 of the Wall reference demonstrates that transgene efficiency ranges from 1% in farm animals (cattle, sheep, pigs) to 3% in laboratory animals, such as rabbits, mice, and rats. This

Table supports Applicants' contention that the generation of transgenic mammals was routine and predictable at the time of the invention, and that methods of transgene expression were available in a wide variety of mammals.

To specifically address the Examiner's concern that the genetic elements required for appropriate expression vary from species to species, Applicants point out that genetic elements that lead to successful secretion of polypeptide from milk and urine were described in the specification and were also well known in the art prior to Applicants' filing date, as addressed above.

Furthermore, nucleic acid constructs for introducing a heterologous gene into a cell or embryo, as well as techniques for generating transgenic animals that produce desired polypeptides, were well known prior to the filing of the present application. These same methods can be relied upon to predictably generate transgenic animals and cultured cells that secrete biofilaments.

In light of the above, Applicants respectfully request that the rejection of claims 1 and 3-21, under 35 U.S.C. § 112, first paragraph, be withdrawn.

Claims 13 and 15-21 also stand rejected under 35 U.S.C. § 112, first paragraph, with the Examiner stating that the specification fails to provide an enabling disclosure for the preparation of any and all species of transgenic animals by the methods recited in the claims.

With respect to this portion of the § 112 rejection, the Examiner specifically states that claims 13 and 15-21 are rejected because the guidance offered in the specification is limited to the generation of mice. The Examiner explains that the claimed methods involve transfecting an embryonal cell, for example, an ES cell or an oocyte, but that transfection of an oocyte is not taught in the specification or the prior art. The Examiner thus concludes that since ES cells technology was known only for the mouse, claims 13 and 15-21 should be limited to the generation of transgenic mice.

In response to this portion of the § 112, first paragraph rejection, Applicants point out that claim 13 has been amended to recite a method for producing a biofilament, comprising providing an embryonal cell <u>transformed</u> with a biofilament-encoding nucleic acid molecule that expresses and causes secretion of the biofilament from a milk-producing or urine-producing cell derived from the transformed embryonal cell; growing the transformed embryonal cell to produce an animal comprising biofilament expressing and secreting cells; and isolating the biofilament from the biofilament-expressing and secreting cells from the animal. Support for this amendment is found in the specification at page 6, line 25 to page 7, line 3, pages 19-20, and pages 26-29. Applicants also note that transformation, for example, by microinjection techniques, of embryonal cells of a number of different species was well known in the art prior to Applicants' filing date. As claims 13 and 15-21 are no longer limited to ES cells technology, Applicants submit that the generation of transgenic animals other than mice would not require undue experimentation.

Claims 13-21 were also rejected based on the assertion by the Examiner that the specification fails to teach a method for producing a biofilament in a transgenic animal by expressing the biofilament in a cell or tissue other than a milk-producing or urine-producing cell.

Applicants have amended claim 13 to recite a method for producing a biofilament wherein the first step is to provide an embryonal cell transformed with a biofilament encoding nucleic acid molecule that expresses and causes secretion of the biofilament from a <u>milk-producing or urine-producing</u> cell derived from the transformed embryonal cell.

As this rejection relates to claim 14 specifically, Applicants contend that the specification provides ample guidance for the production of biofilaments from cultured cells (see, for example, page 21, lines 4-7 and pages 25-26). Methods for the production

of polypeptides secreted from cultured cells were also known in the prior art (e.g., U.S. Patent No. 5,227,301, previously submitted in an Information Disclosure Statement). Accordingly, Applicants submit that undue experimentation would not be required to practice the invention of claims 13 and 15-21, and respectfully request that this rejection be withdrawn.

Claims 2-6 were also rejected under 35 U.S.C. § 112, first paragraph, on the ground that the written description requirement is not satisfied for the claimed genus. Specifically, the Examiner states that a representative number of species have not been described by their complete structure.

In response to this rejection, Applicants note that claims 3-6 have been amended to recite a phenotype for the claimed transgenic animals. Applicants submit that these claim amendments provide relevant identifying characteristics for the transgenic animals, and satisfies the written description requirement. Applicants also point out that one in possession of a transgenic animal of claims 3-6, would also have been in possession of the embryo of claim 2, as it is used to generate the transgenic animal. Withdrawal of this rejection is respectfully requested.

Rejections Under 35 U.S.C. § 112, second paragraph

Claim 2 was rejected U.S.C. § 112, second paragraph, based on the assertion by the Examiner that the claim is indefinite in its recitation of "whose nucleus" in reference to an embryo. The Examiner states that an embryo itself does not have a nucleus. In response to this rejection, Applicants have amended claim 2 to recite an embryo comprising a cell whose nucleus comprises the nucleic acid molecule of claim 1. This rejection may be withdrawn.

Claims 3 and 4 were also rejected under U.S.C. § 112, second paragraph, with the Examiner stating that the claims are indefinite in their recitation of "[a] female mammal

in which the genome of the mammary tissue of said female comprises the nucleic acid molecule of claim 1" because it is unclear as to which cells of the transgenic animal are transgenic. The Examiner further states that transgenic animals carry a transgene in the genome of all somatic and germ cells.

In response to this rejection, Applicants have amended claim 3 to recite a non-human female transgenic mammal in which the genome of the mammary tissue of the female mammal comprises the nucleic acid molecule of claim 1, wherein the promoter is milk-producing cell-specific, and wherein the mammal secretes the biofilament of claim 1. With the addition of the term "transgenic" to claim 3, Applicants submit that the claim (and dependent claim 4) clearly recites a female mammal in which the genome of every cell comprises a transgene. Withdrawal of the rejection is respectfully requested.

Claims 5 and 6 were also rejected under U.S.C. § 112, second paragraph, on the ground that claim 5 broadens the scope of claim 1 by being directed to a transgenic animal comprising the nucleic acid molecule of claim 1, a nucleic acid molecule that encodes and secretes a biofilament into the milk or urine of a mammal. This rejection has been met by the amendment of claim 5 to recite a non-human transgenic mammal in which the genome of cells that contribute to urine production in the animal comprises the nucleic acid molecule of claim 1, wherein the promoter is urine-producing cell-specific, and wherein the mammal secretes that biofilament of claim 1.

Claims 13 and 15-21 were rejected under U.S.C. § 112, second paragraph, based on the assertion by the Examiner that the claims recite a method for producing a biofilament in which the first step recites providing "an embryonal cell transfected with a biofilament encoding nucleic acid molecule." The Examiner notes that no methodology exists for transfecting a fertilized oocyte. As discussed above, the rejection has been met by the amendment of claim 13 to recite "an embryonal cell transformed with a biofilament encoding nucleic acid molecule."

Claims 14 and 15-21 are also rejected under U.S.C. § 112, second paragraph, with the Examiner stating that the nucleic acid molecule of the claim does not recite that the promoter is operably linked to the nucleic acid sequence encoding the biofilament. This rejection has been met by the amendment of claim 14, reciting that the promoter is operably linked to the nucleic acid sequence encoding the biofilament.

Claims 14-21 also stand rejected under U.S.C. § 112, second paragraph, on the ground that the recited methods are incomplete because they omit the essential step of the expression and secretion of the biofilament by the transformed cell. Such amendment has been made to claim 14, and this rejection can now be withdrawn.

Finally, Applicants note that there have been no rejections based on prior art.

CONCLUSION

Applicants submit that the claims are in condition for allowance, and such action is requested. Enclosed is a petition to extend the period for replying for three months, to and including October 12, 2000. If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: October 12, 2000

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Exhibit A

RESEARCH

HIGH LEVEL EXPRESSION OF ACTIVE HUMAN ALPHA-1-ANTITRYPSIN IN THE MILK OF TRANSGENIC SHEEP

G. Wright[1], A. Carver[1], D. Cottom, D. Reeves, A. Scott, P. Simons[2], I. Wilmut[2], I. Garner, and A. Colman*

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We describe the generation of five sheep transgenic for a fusion of the ovine β-lactoglobulin gene promotor to the human α_1-antitrypsin (hα_1AT) genomic sequences. Four of these animals are female and one male. Analysis of the expression of hα_1AT in the milk of three of these females shows that all express the human protein at levels greater than 1 gram per liter. In one case initial levels exceeded 60 grams per liter and stabilized at approximately 35 grams per liter as lactation progressed. Human α_1AT purified from the milk of these animals appears to be fully N-glycosylated and has a biological activity indistinguishable from human plasma-derived material.

The prospect of producing large quantities of therapeutic proteins in the milk of transgenic livestock was publically raised some years ago[1,2]. As an alternative to cell culture systems, this production route is appealing because of the simplicity of access to the expressed protein, the high production capabilities of the mammary gland, the relatively low operating costs, and finally, the potentially unlimited expansion of the producer animals through established and emerging methods of animal husbandry. Like expression systems based on cultured mammalian cells, the mammary gland appears to be capable of performing the post-translational modifications vital to the activity or stability of certain pharmacologically active human proteins. In the last five years numerous publications have appeared attesting to the feasibility of this approach (for review, see ref. 3). Typically the gene of interest (either cDNA or genomic DNA) is fused to the regulatory sequences of the gene for a milk protein, and the fusion construct used to generate transgenic animals. In numerous cases the desired protein has been found in the milk, however the yields of protein have been extremely variable and usually much less than 1 gram per liter, although levels as high as 23 grams per liter in mice[4] and 2 grams per liter in pigs[5] have been obtained with foreign milk proteins, and 1–2 grams per liter of human urokinase, a plasminogen activator, has been produced in mouse milk[6].

Human α_1-antitrypsin (hα_1AT) is a 394 amino acid glycoprotein which is normally present at 2 grams per liter in plasma[7]. The primary site of hα_1AT production in the body is the liver[7–9], and genetic deficiencies in circulating concentrations of hα_1AT are one of the most common lethal hereditary disorders to affect Caucasian males of European descent and sufferers are at risk of developing life-threatening emphysema. Replacement therapy using human plasma-derived α_1AT, has been sanctioned in the USA[7] where the large number (>20,000) of affected individuals and large amounts needed (~200 grams/patient/year[10]) make a strong case for an alternative, recombinant DNA-derived source, which is capable of yielding large quantities of hα_1AT and of performing the glycosylation events needed for plasma stability[10]. Recently Archibald et al.[11] reported yields of up to 7 grams per liter of biologically active hα_1AT in the milk of transgenic mice expressing a minigene containing the sheep betalactoglobulin (BLG) promotor fused to hα_1AT sequences, which comprise part of exon 1 and the remaining, downstream introns and exons, excluding intron 1. If similar high yields could be obtained in the milk of transgenic livestock, this could form the basis for a manufacturing process.

In this paper we demonstrate that the sheep mammary gland can offer a production route for large quantities of glycosylated, bioactive hα_1AT. Using the hα_1AT minigene described above, we report the generation, lactation, and milk protein analysis of three founder transgenic animals. Two of the animals produce 1–5 grams per liter quantities of hα_1AT whilst the third produces ~35 grams per liter making hα_1AT the major protein in the milk. These levels have been sustained in all cases through 7 weeks of lactation and exceed by several orders of magnitude previously reported yields of foreign proteins in sheep milk and, in one case, the yield is substantially (>17 fold) higher than that reported for any foreign, non-milk protein in any transgenic system[3]. We believe these results confirm the feasibility of using mammals as bioreactors for the production of human therapeutic proteins.

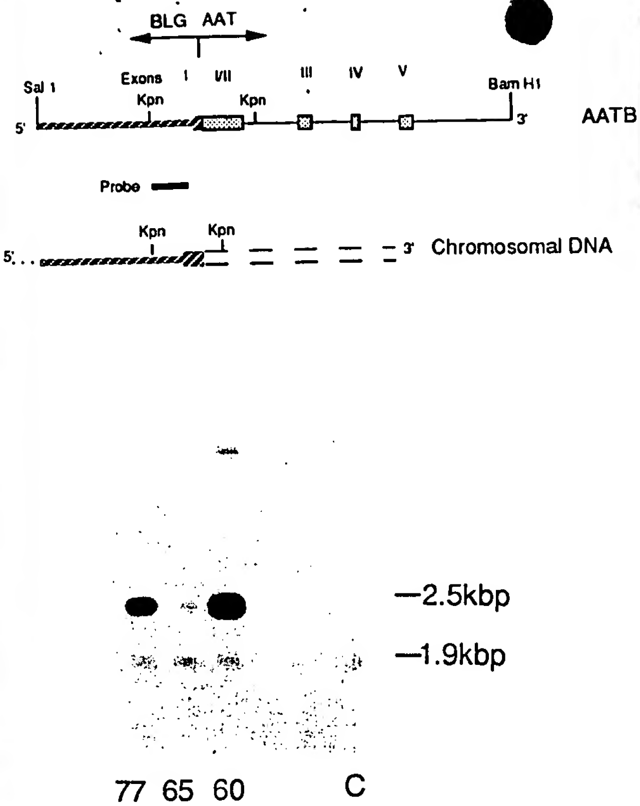

FIGURE 1 Southern blot analysis of transgenic DNA. The diagram shows the relevant region of the sheep chromosomal BLG locus as well as the intron/exon structure of the AATB construct used for injection (also see ref. 11); hatched regions correspond to BLG sequences and stippled regions and plain lines correspond to AAT sequences. DNA purified from the peripheral blood lymphocytes of transgenic sheep 60, 65 and 77 and a non-transgenic control animal (C) was digested with KpnI and analyzed as described in the Experimental Protocol. The membrane was hybridized with a radioactive probe homologous to 800 bp of the ovine BLG promoter (see diagram) and was generated by random priming (Stratagene). This reveals a 2.5 kbp internal band from intact transgenes and a 1.9 kbp band derived from endogenous BLG sequences (see diagram).

TABLE 1 Summary of the generation of transgenic sheep

Parameter	Value
No. eggs injected	549
No. eggs surviving	439
No. recipients	152
No. eggs per recipient	2.88
No. of pregnancies	73
No. births	113
No. screened	112*
No. transgenic	5
Percent of births transgenic	4.5
Percent of injected eggs transgenic	0.91

*One animal was stillborn and proved unsuitable for analysis.

RESULTS

Generation of transgenic sheep. Archibald et al.[11] recently described the production of bioactive hα_1AT in the milk of mice transgenic for a hybrid ovine BLG-hα_1AT gene, referred to as AATB. Of seven lines of animals expressing the AATB transgene at variable levels in the lactating mammary gland, four produced milk levels of greater than 0.5 grams per liter hα_1AT with one yielding a level in excess of 7 grams per liter. For this study, we made use of the same hybrid AATB construct.

Initially, we extended the observations of Archibald and colleagues by generating nine G_0 founder mice transgenic for the AATB fusion. Although levels of hα_1AT produced in the milk of these animals varies from line to line, all express the transgene at between 0.4 mg and 12.45 grams per liter. Moreover, the highest expressing animal has transmitted the transgene to her offspring and all G_1 females (three) exhibit a capacity similar to that of their mother to secrete hα_1AT with their milk (unpublished data).

These studies suggest that the AATB construct is efficient at directing the expression of hα_1AT to the lactating mammary gland with concomitant secretion of the human protein. To confirm that this is true not only in mice but also in sheep, we generated sheep transgenic for the AATB fusion gene. A total of 549 sheep eggs were microinjected with purified AATB DNA giving rise to 113 lambs (Table 1). One of these animals was stillborn and proved unsuitable for further analysis. Of the remaining 112, five proved to be positive for the AATB hybrid gene upon Southern blot analysis of genomic DNA samples. Four of these are female and one male.

These five animals developed normally and have shown no ill effects attributable to the presence of the transgene. To date, three of the females (nos. 60, 65 and 77) have produced offspring. Sheep 60 produced two female lambs, one of which is transgenic, sheep 65 produced one non-transgenic male and sheep 77 produced one non-transgenic female (data not shown). To assess the integrity of the incorporated transgenes in these three G_0 animals we performed Southern blot analyses of genomic DNA derived from peripheral blood lymphocytes. Cleavage of integrated copies with KpnI should release an internal fragment of 2.5 kbp (Fig. 1). This is revealed with a probe covering the first 800 bp of the BLG sequences present in AATB. The probe also reveals a 1.9 kbp band derived from endogenous ovine BLG sequences by hybridization to identical target sequences. Comparisons of band intensities with copy number controls (data not shown) and the endogenous BLG bands suggest that sheep 60 contains ~10 copies of the transgene, sheep 65 ~2 copies and sheep 77 ~5 copies. Analyses using other restriction enzymes and probes suggest that the multiple integrants contain intact copies of the transgene (data not presented). However, as previously found in transgenic sheep[12], the arrays are complex with both head to head and head to tail repeats. The elucidation of the exact structure of these arrays awaits further study.

Levels of human α_1AT in transgenic sheep milk. The offspring from animals 60, 65 and 77 were artificially reared and milk collected daily from their lactating mothers. Samples were pooled on a weekly basis and analyzed for the presence of hα_1AT. Initial determinations were performed with both a radial immunodifusion assay (RID) and ELISA. Neither of these techniques produce a cross-reaction with sheep α_1AT. A good correlation was observed between results obtained with these techniques and further determinations were performed using RID alone.

Levels of hα_1AT present in the milk of all three founder animals have exceeded 1 gram per liter (Table 2). There is no direct relationship between transgene copy number and levels of expression. It is notable, however, that yields do increase with increasing copy number. Sheep 60 produced 63 grams per liter hα_1AT in week one but has since stablized to yield ~35 grams per liter in

subsequent weeks. The human protein is consistently ~50% of the total protein in the milk of this animal. Sheep 65 produced 3.8 grams per liter in week one and has since stablized at around 1.5 grams per liter. Again this is a constant percentage of the total protein produced of about 3.5%. In contrast, Sheep 77 began secreting hα_1AT at 0.9 grams per liter and has since increased output attaining 3.5 grams per liter in week seven. This reflects an increase in the percentage of total protein that is hα_1AT from 1.4 to 10%. We have no explanation for this at present.

It should be noted that milk from week one contained colostrum and as such had higher concentrations of both hα_1AT and total protein. However, the total protein levels recorded for subsequent weeks has remained within observed limits for sheep milk despite being higher than expected for this breed (Blackface/Friesland). We are, therefore, not in a position to comment on whether endogenous protein production has been suppressed in these animals or whether total protein production has been increased.

Characterization of human α_1AT from transgenic sheep milk. Milk from founder animals 65, 77 and 60 was analyzed by SDS/PAGE (Fig. 2, lanes 2, 3 and 4 respectively). A novel band of apparent 54 kD molecular weight was observed in all three samples (indicated by arrow). This is the predicted molecular weight of native plasma derived hα_1AT (Fig. 2, lane 8). We confirmed this to be hα_1AT by western blotting (data not shown). Note that in the sample derived from sheep 60, the hα_1AT is the major protein in the milk.

Milk samples from all three sheep were defatted and hα_1AT was purified from the remaining material using anion exchange, dye affinity, hydrophobic interaction and gel filtration chromatography (manuscript in preparation). When analyzed by reducing (data not shown) and non-reducing SDS/PAGE (Fig. 2, lanes 5, 6 and 7) all three products migrate as a single band of about 54 kD similar to that observed for plasma derived hα_1AT (Fig. 2, lane 8). We estimate the purity of the three products to be >95% following silver staining, densitometry scanning and HPLC analysis (data not shown). Sheep milk naturally contains 1–2 µg per ml α_1AT. Our purified material could therefore contain a small percentage of sheep α_1AT that would not be revealed by our RID or ELISA assays, which are specific for the human protein. However, a comparison of the results obtained from these two techniques with total protein estimates indicated that our purified hα_1AT is at least 95% human protein. This is supported by amino terminal sequence data, which do not reveal any contamination with sheep α_1AT (manuscript in preparation).

Glycosylation of human α_1AT from transgenic sheep milk. Human α_1AT has three N-linked branched carbohydrate chains linked to asparagines 46, 83 and 247. Non-glycosylated recombinant hα_1AT is active but exhibits an accelerated *in vivo* plasma clearance[10], probably reflecting the absence of carbohydrate moieties. The apparent molecular weight of the material purified from transgenic sheep milk suggests that it is fully glycosylated, and to determine if this is so samples were cleaved with N-glycosidase F (Fig. 3). Lanes 1–4 contain uncleaved material and lanes 5–8 cleaved samples. In all cases, the hα_1AT purified from transgenic sheep milk behaves similarly to hα_1AT purified from human plasma (Fig. 3, lanes 1 and 8). Digestion of all samples results in a shift of electrophoretic mobility similar to that observed with human plasma derived α_1AT (Fig. 3, lanes 1 and 8). Furthermore, all of our purified material appears to be fully N-glycosylated (compare lanes 2–4 with lanes 6–8). Re-examination of the hα_1AT from complete milk (eg.

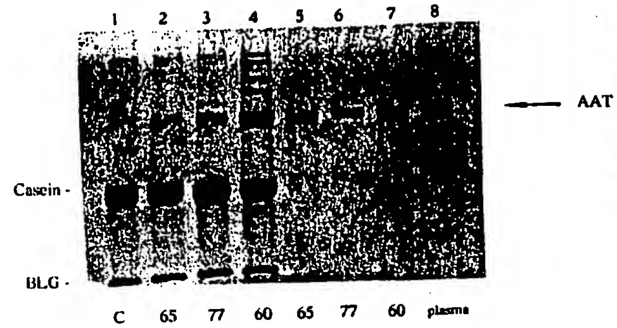

FIGURE 2 Non-reducing SDS-PAGE of transgenic sheep milk and purified hα_1AT. Aliquots (0.1 µl) of whole milk from transgenic sheep 65, 77, 60 and a control (C) non-transgenic animal (lanes 1–4) or 1 µg of hα_1AT purified from the milk of transgenic sheep (lanes 5–7) were analyzed on a 12% non-reducing, SDS-PAGE gel as described in the Experimental Protocol. Lane 8 contains 1 µg hα_1AT purified from human plasma (Miles, Inc.). The running positions of casein and betalactoglobulin are indicated on the left of the figure. The position of plasma derived hα_1AT is indicated by the arrow on the right of the figure.

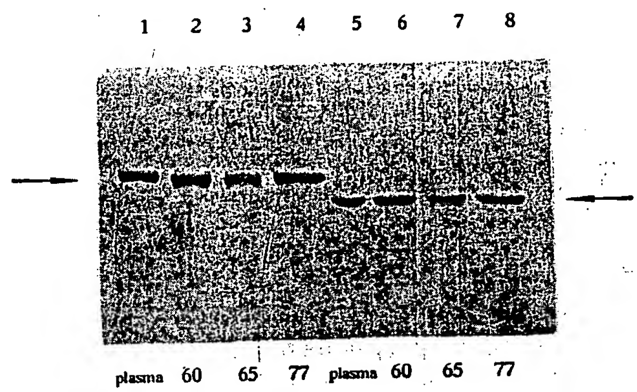

FIGURE 3 SDS-PAGE of glycosylated and deglycosylated hα_1AT. hα_1AT (0.5 µg) samples purified from human plasma (Miles, Inc.) or from the milk of transgenic sheep 60, 65 and 77 were treated with (lanes 5–8) or without (lanes 1–4) N-glycosidase F as described in the Experimental Protocol. The arrow to the left of the figure indicates the position of glycosylated material (54 kD), the arrow to the right indicates the position of cleaved deglycosylated material (45 kD). MW markers are not shown.

TABLE 2 Analysis of human α_1AT in transgenic sheep milk.

Sheep	Week	Protein Content	h α_1AT Content
60	1	127.2	63.0
	4	66.8	31.7
	7	71.2	37.5
65	1	72.4	3.8
	4	44.1	1.3
	7	41.6	1.5
77	1	64.0	0.9
	4	44.4	2.2
	7	35.8	3.5

Animals were milked daily and the weekly produce was pooled prior to analysis. Figures from three representative weeks are presented in grams per liter of milk.

track 4, Fig. 2) shows that it corresponds in mobility to the glycosylated products shown in Figure 3 and therefore excludes the possibility that the purification was selective for glycosylated forms of α_1AT, indicating that all the hα_1AT is glycosylated. These results demonstrate the ability of the ovine mammary gland to N-glycosylate large quantities of secreted protein. We are currently determining the nature of these sugar moieties.

Bioactivity of hα_1AT purified from transgenic sheep milk. To analyze the activity of our purified hα_1AT we compared its ability to inhibit trypsin to that of two samples of plasma-derived hα_1AT using a colorimetric assay. A standard curve generated with one plasma-derived source was used to determine the activities of each of the other samples. In all cases, the hα_1AT purified from transgenic sheep milk shows similar activity to both plasma-derived products (Table 3).

DISCUSSION

We report in this paper the production in sheep milk of large amounts of a foreign protein, human α_1AT. We presume that this protein is made predominantly in the mammary gland for several reasons. First, analysis of the tissue-specificity of transcription from the AATB construct in mice indicates that the major site of transcription is the mammary gland, although in some animals a low level of expression from the salivary gland was noticed[11]. Second, if hα_1AT were synthesized outside the mammary gland, it would presumably gain access to the mammary gland via the blood. However circulating levels of hα_1AT are negligible compared to sheep α_1AT whereas this situation is reversed in the milk (data not shown). Direct analysis of RNA expression will be performed eventually, however at present we are concerned not to compromise the animals during their first lactation.

Concentrations up to 35 grams per liter of hα_1AT have been obtained. This level of hα_1AT production has now been sustained throughout the lactation period (twelve weeks); this situation contrasts with that recently reported for transgenic swine producing mouse whey acid protein where lactation itself was not sustained in 2 out of 3 lines as a result of transgene expression[5]. With milk yields per lactation ranging from 250–800 liters, according to sheep breed, the overall yield of hα_1AT per animal per lactation could exceed 10 kg. The hα_1AT recovered shows similar bioactivity to the human plasma-derived product. In addition, the hα_1AT produced by all three animals is fully N-glycosylated and we are presently investigating the exact sugar composition of the carbohydrate side chains. This demonstrates that in the mammary gland, the glycosylation apparatus has not been saturated by the requirement for hα_1AT glycosylation even though normally only a small proportion of the endogenous milk protein (<10%) is glycosylated and most of this represents O-linked glycosylation of κ-casein. Despite the commandeering of the animals' transcriptional and translational machinery for foreign protein production, all the transgenic sheep described here are perfectly normal and healthy. Although we have so far only been able to demonstrate transgene transmission in one of the three female sheep [the one transgenic male, has transmitted the transgene (data not shown)], the seven out of eight transgenic sheep previously generated by Clark and colleagues[12] have been found to transmit their transgenes in an unrearranged fashion (J. Clark, personal communication).

With few exceptions[13–15] it still remains the case that expression from the same transgene construct is highly variable between different lines. This has been attributed to various causes, including host genetic background, site(s) of chromosomal insertion, absence of certain transcriptional elements, etc[16]. Although there is no formal proof, we believe that the sheep BLG gene used to provide control elements for our transgene constructs has all the regulatory sequences necessary to confer high expression on a foreign gene fragment, since expression of the complete BLG gene in transgenic mice led to a range of yields but nearly all of them were high[4]. Although dramatically lower expression levels have been reported for fusion constructs between foreign genes and milk protein gene promotors including sheep BLG, this may be attributable in part to the absence of native introns in the foreign gene inserts[17]. Improvements in expression have been obtained when native, foreign or hybrid introns are added back[17–19]. When originally expressed in mice by Archibald et al.[11], the minigene used in our study gave hα_1AT yields of 80 milligrams −7.7 grams per liter with some animals not producing any detectable protein at all. Repeating this work we obtained a range of yields from 0.4 milligrams −12.45 grams per liter from nine different lines, a 30,000-fold range in variation. Although only 3 founder ewes have been analyzed in the study reported here (the fourth ewe is about to give birth), a 10-fold range of yields was obtained. While a comparison of the mice and sheep hα_1AT yields is questionable due to the small sample size, it is notable that the expression levels in sheep are on average higher and less variable. This may be a consequence of the homologous combination of an introduced sheep milk protein gene promotor operating in a sheep mammary gland environment.

In summary, we describe the production of high levels of a human therapeutic protein, α_1AT, in sheep milk. In one case the hα_1AT represents nearly 50% of total milk protein throughout the lactation period. These results indicate that it is possible to dramatically alter milk composition, opening up opportunities in the dairy industry to carry out a range of manipulations from over-expression of existing proteins to the introduction of novel milk proteins, which may allow improvements in milk formulations for both adult and infant consumption. In addition, this level of hα_1AT production exceeds those obtained in bacteria (15% total cell protein[20,21]), yeast (40% soluble protein[22]), and cultured mammalian cells (<1mg/10^6 cells/24h[23]), and provides a strong impetus to the further exploitation of transgenic sheep as bioreactors for the production of large amounts of pharmacologically active proteins.

TABLE 3 Bioactivity of hα_1AT purified from transgenic sheep milk.

Source of Purified hα_1AT	Percent Activity
Sheep 60 milk	95 ± 12
Sheep 65 milk	93 ± 22
Sheep 77 milk	86 ± 15
Human plasma α1AT (Miles, Inc.)	100
Human plasma α1AT (Sigma)	94 ± 5

Results are derived from 5 separate assays performed on two separate days and are normalized to the values obtained with the Miles, Inc. sample.

EXPERIMENTAL PROTOCOL

Generation of transgenic sheep. Transgenic sheep were generated essentially as described by Simons et al.[12] with the following differences: superovulation was induced with regimes of equine, porcine or ovine FSH; ovulation was synchronized in donor ewes (Scottish Blackface) using Receptal (Hoechst Animal Health); eggs were collected from donor ewes artificially inseminated with approximately 10^7 fresh, motile spermatozoa (Friesland) by intrauterine laparoscopy; eggs were collected by mid-ventral laparoptomy

approximately 17 hours after the expected mod[illegible]e of ovulation.

DNA preparation and analysis. Peripheral blood lymphocytes were prepared from transgenic sheep blood using Histopaque (Sigma) according to the manufacturers instructions. Genomic DNA was prepared by proteinase K (BCL) digestion and phenol extraction. Following digestion with appropriate restriction enzyme(s), samples were subjected to electrophoresis in 1% agarose gels, transferred to Duralon trademark membranes and hybridized to radioactive probes as described by the manufacturer (Stratagene).

Gel analysis of protein samples. Milk and purified hα_1AT samples were diluted in 75 mM-Tris/HCl buffer at pH 6.8, containing 2.5% (w/v) SDS and 10% (w/v) glycerol. These samples were boiled for 2 minutes and then electrophoresed on 12% discontinuous SDS polyacrylamide gels[24]. After running, gels were stained with 0.125% Coomassie blue R-250 in a 50% methanol/10% acetic acid solution and destained with the solvent alone.

Measurement of human α_1AT. Concentrations of hα_1AT were measured by radial immunodiffusion (RID) and confirmed by enzyme linked immunosorbant assay (ELISA). RID plates were obtained from the Binding Site, and hα_1AT levels were measured using the method described in the manufacturer's instructions. For the ELISA assay, polyclonal rabbit anti-human hα_1AT antibodies (Dako) were diluted 1/1000 in 0.1 M-$NaHCO_3$, pH 8.2. Aliquots (250 µl) of this solution were added to each well of microtiter plates and then these were incubated overnight. The next day the plates were washed, and various sample dilutions in 1/1000 normal rabbit IgG (Dako) were added to the wells. The plates were incubated for 2.5 hours before adding 1/1000 diluted biotinylated polyclonal rabbit anti-human hα_1AT second antibodies. The plates were then incubated for 90 minutes before being washed and adding a streptavidin/biotin-horseradish peroxidase conjugate (Boehringer). This was followed by another 90 minute incubation before the plate was again washed and finally 100 µl of the substrate solution 1,2 phenylenediamine and 0.01% (v/v) hydrogen peroxide was added. Color was allowed to develop and this was measured at 492 nm. The hα_1AT content of samples was measured by comparing the 492 nm results to those obtained with standards containing known amounts of hα_1AT. Except for the initial step where the antibodies were bound to the plate, all washes were carried out, and dilutions made, in phosphate buffered saline containing 5% bovine skimmed milk; 0.1% Tween 20; and 0.1 mM-EDTA. All incubations were at room temperature in a moisture chamber. The second antibodies were biotinylated by incubating them in 50 mM-Tris/acetate buffer at pH 7.5 for 4 hours with 0.8 mM biotinyl-ϵ-amino caproic acid N-hydroxysuccinimide ester and then dialyzing against the above buffer alone. Plasma derived samples of hα_1AT were purchased from Sigma or were the kind gift of Miles, Inc. (Berkeley, CA).

Deglycosylation of human α_1AT. Samples of purified hα_1AT (0.5 mg/ml) were suspended in 100 mM-sodium phosphate buffer at pH 7.5 containing: 25 mM-EDTA; 1% *n*-octylglucoside; 1% 2-mercaptoethanol; and 0.1% SDS. These were boiled for 5 minutes, cooled to room temperature, and then 10 units/ml of N-glycosidase F (Boehringer) was added. Samples were then incubated for 16 hours at room temperature before they were run on SDS-PAGE. Control samples of hα_1AT were treated in exactly the same way, except no N-glycosidase was added.

Bioassay of human α_1AT. The hα_1AT activity assay used is based on the affinity of human α_1AT for trypsin. Nα-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), in the presence of trypsin (Sigma), breaks down to benzoylarginine and the colored compound *p*-nitroanalide, the absorbance of which can be measured at 405 nm. Dilutions of purified hα_1AT (130 µl) were incubated at room temperature with 50 µl of 0.25 mg/ml porcine trypsin type II (Sigma) in 50 mM-Tris/acetate buffer at pH 7.5 for 5 minutes before the addition of 20 µl of the chromogenic substrate BAPNA (Sigma). After 15 minutes the absorbances at 405 nm were read. A standard curve using constant amounts of trypsin (62.5 µg/ml) and BAPNA (1.5 mM), and varying amounts of human plasma-derived α_1AT (5–40 µg/ml; Sigma) was constructed. The inhibition of the same amount of trypsin/BAPNA by purified hα_1AT samples was then translated into a percentage activity relative to the purified plasma-derived hα_1AT (Miles, Inc.).

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Note added in proof: The fourth ewe transgenic for AATB has given birth to a female lamb that has inherited the transgene. The mother is expressing hα_1AT in her milk at a level of 3.2 g/l, which further reinforces our view that high level expression from this construction in the milk of transgenic sheep is the norm rather than the exception.

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Production of Biologically Active Human Protein C in the Milk of Transgenic Mice[a]

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INTRODUCTION

There are many therapeutic proteins derived from human plasma and their availability is dependent upon the limited supply of plasma.[1] As a result, extensive efforts are being made to produce recombinant versions of these proteins.[2-7] Recent advances in the understanding of gene expression have led to high level production of relatively simple proteins in both cell culture[8,9] as well as the mammary gland of transgenic mice[10-12] and livestock.[13-16] However, these expression systems have not yet demonstrated the ability to perform many aspects of protein processing needed to synthesize highly complex enzymes.[10-17] For example, proteolytic cleavage of propeptide sequences and amino acid derivatization are frequently essential for efficient secretion or biological activity.[2,6] The present study has focused upon the vitamin K-dependent (VKD) carboxylation of glutamic acid, which has not been shown previously to occur in mammary tissue to any significant level.[6,17]

Multiple gamma-carboxylated glutamic acid (gla) residues are necessary for the membrane-mediated proclotting and anticlotting activities of the VKD proteins.[18-21] The carboxylase activity necessary for gla formation has been directly or indirectly found to be limited in most tissues[22,23] and manipulated cell lines.[2,4] As a result, biologically active VKD proteins have been produced in genetically engineered

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systems at levels of less than 0.4 μg/mL/hour.[2-6] Furthermore, the isolation of most VKD proteins from human plasma is difficult because they occur at less than 10 μg/mL, with the exception of prothrombin, which is found at approximately 120 μg/mL.[24] Alternatively, if higher levels are to be produced in cell culture, greater levels of carboxylase activity may be needed for the efficient secretion of biologically active VKD proteins.[2-4]

Human Protein C (hPC) is a member of the VKD protein family and serves as the central regulator of hemostasis.[21] Therefore, hPC has potential as a therapy for many disease states. Examples include fibrinolytic therapy,[21] vascular trauma (as occurs in surgical procedures such as hip and knee replacement),[21] congenital deficiency of hPC,[25] and blood poisoning.[26] Protein C exists as the zymogen of a serine protease that undergoes activation by thrombin.[27] Activated Protein C inhibits further generation of fibrin clots by proteolytic cleavage of factor VIIIa and factor Va.[28] Although several different forms occur in human plasma, the most prevalent hPC form consists of a 62,000-M_r glycoprotein with 4 N-linked glycosylation sites, 12 intrachain disulfide bridges, and 1 beta-hydroxylated aspartic acid residue.[29] The mature zymogen structure results from proteolytic cleavages of prepro-hPC that remove signal and propeptide sequences that have been predicted to be 33 and 9 amino acids in length, respectively.[29] In 70–95% of plasma-derived hPC, a dipeptide at amino acids 156–157 has also been removed to yield a heterodimeric form of hPC consisting of a 41,000-M_r heavy chain and a 21,000-M_r light chain[27] linked by a single disulfide bridge.[30,31] Within the first 29 amino acid residues of the light chain, there are 9 gla residues that are essential for the anticlotting function of hPC.[20,27,29]

The structure and function of hPC make it one of the most complex members of the VKD protein family and efforts to express Protein C in recombinant cell lines have had limited success.[2,5-7] Of the many cell lines, including those derived from human liver[5] and mouse mammary tissue,[6] only the human kidney 293 cell line[2,32] has produced fully functional recombinant Protein C (rhPC) at 1–25 μg · (10^6 cells)$^{-1}$ · (24 hours)$^{-1}$. However, the rhPC produced by the 293 cells had both structural and functional properties that differed from those of plasma-derived hPC.[2,32] We present a study that demonstrates the ability of murine mammary tissue to produce biologically active rhPC that closely resembles the native population of hPC.

MATERIALS AND METHODS

DNA Construct

Plasmid containing the murine whey acidic protein–human Protein C (WAPPC-1) hybrid gene was received as a gift from Christoph Pittius and Lothar Hennighausen (Molecular Genetics Laboratory, NIH, Bethesda, Maryland). The WAPPC-1 construct (FIGURE 1) was purified by digesting plasmid DNA with restriction endonuclease *Eco* RI. The WAPPC-1 construct was purified from the plasmid DNA using a GEN-PAC FAX (Millipore Corporation, Milford, Massachusetts) high performance liquid chromatography (HPLC) column. The elution conditions for the WAPPC-1 product were determined by gradient chromatography over the range of 0.5–1.0 M NaCl. An isocratic elution condition was chosen (25 mM TRIS-HCl, 1.0 mM EDTA, and 0.63 M NaCl, pH 7.5). Approximately 15–20 μg of digested DNA was injected

per run and eluants containing the WAPPC-1 fragment from each injection were pooled, precipitated, and processed by HPLC a second time. The purity and concentration of WAPPC-1 were determined on a 1% agarose gel stained with ethidium bromide.

Transgenic Mice

Female CD-1 mice (Charles River Laboratories, Wilmington, Massachusetts), 3–4 weeks of age, were superovulated with interperitoneal injections of 10 IU Pregnant Mare's Serum Gonadotropin (Diosynth Incorporated, Chicago, Illinois) followed by 5 IU human Chorionic Gonadotropin (hCG; Sigma Chemicals, St. Louis, Missouri) 48 hours later and these were then placed with stud males. Embryos were then collected 21–23 h after hCG. One-cell embryos were microinjected with 1–3 pL

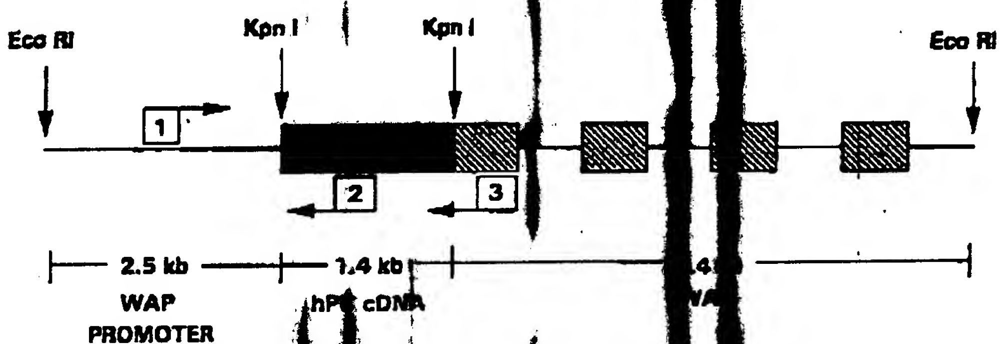

FIGURE 1. Schematic diagram of the whey acidic protein–human protein C fusion gene. The cDNA for hPC was inserted into the *Kpn* I restriction site present just before exon 1 of WAP. The boxes marked 1, 2, and 3 schematically represent the relative sequence positions of oligonucleotide primers. Solid lines represent noncoding WAP DNA and/or WAP introns, the solid box represents cDNA encoding hPC, and hatched boxes indicate WAP exons. Priming of DNA synthesis with three oligonucleotides as indicated permits simultaneous detection of both the transgene and the endogenous WAP gene in transgenic mice. Only the smaller target sequence (primed by oligonucleotides 1 and 3) is detected in nontransgenic mice.

of DNA solution (3.3 μg/mL DNA, 10 mM TRIS-HCl, and 0.25 mM EDTA, pH 7.4) according to Brinster *et al.*[33] Surviving embryos were transferred to pseudopregnant females (20–30 embryos per recipient).

DNA Extraction

Tail tissue was biopsied from pups that were approximately 20 days old and DNA was isolated from the tissue by a modification of the procedure developed by Marmur.[34] Briefly, 840 μL of lysing solution (50 mM TRIS-HCl, 0.15 M NaCl, 1 M Na_2ClO_4, 10 mM EDTA, 1% sodium dodecylsulfate, 1% β-mercaptoethanol, 100 μg/mL proteinase K, pH 8.0) was added to each tube containing a tissue sample that had been previously frozen in liquid nitrogen. The tubes were incubated overnight at 50 °C and then extracted with 250 μL of chloroform/isoamyl alcohol (25:1) by mixing

for 10–15 seconds on a "Mini Bead-Beater" (Biospec Products, Bartlesville, Oklahoma), followed by centrifugation for 10 minutes at 15,000g. DNA was precipitated by adding 50 μL of isopropyl alcohol to 83 μL of the aqueous supernatant and this was then centrifuged and washed with 80% ethanol. The pellets were dried at 37 °C, resuspended in 50 μL of TE (10 mM TRIS-HCl and 1.0 mM EDTA, pH 8.0), and stored at −20 °C until assayed by the polymerase chain reaction (PCR).

PCR Analysis

Analysis was done by the general method of Saiki et al.[35] One μL of DNA solution was used as the template in 25-μL reaction volumes [1 × Taq buffer, 2.5 μM dNTP's, 0.5 μM oligonucleotide primers, 0.625 units Taq polymerase (Promega Corporation, Madison, Wisconsin)]. The primers used to amplify a 402-bp target sequence in the transgene were WAP-specific sense 5′-GTC GCC AAG AAG GAA GTG TTG and hPC-specific antisense 5′-GTG CTT GGA CCA GAA GGC CAG. The WAP-specific antisense primer 5′-GAG TTG TTC CTC TAG GTT CTG was also added to amplify a 222-bp fragment contained within the endogenous WAP gene. Initial denaturation was performed at 96 °C for 1 minute followed by 40 cycles of 55 °C annealing for 2 minutes, 77 °C elongation for 75 seconds, and 96 °C denaturation for 15 seconds. Amplification products from mouse tail DNA were run concurrently with those from plasmid DNA on 1% agarose gels stained with 0.5 μg/mL ethidium bromide.

Mouse Milk Collection and Preparation

Females were removed from their pups for approximately 1 hour prior to milking to allow for milk accumulation. These females were then anesthetized with Metofane (methoxyfluorothane; Pitman-Moore, Washington Crossing, New Jersey) and induced to let down milk by intramuscular administration of [illegible].0 IU oxytocin (Vedco Incorporated, St. Joseph, Missouri). Milk was collected into 1.8-mL screw-cap microcentrifuge tubes using capillary tubes (Kimax brand, 2.0-mm i.d.) that were flame-polished to prevent tissue damage. The capillary was partially inserted into a stoppered hand-held receiving chamber containing the microfuge tube. The milk was collected from the capillary directly into the microcentrifuge tube while operating the receiving chamber at 12 cm H_2O vacuum. Upon collection of 150 to 500 μL of milk, the tubes were stored at −90 °C until the final whey preparation stage.

To maximize the recovery of whey-soluble proteins (including Protein C), the whole milk was diluted with three volumes of TBS (50 mM TRIS-HCl and 150 mM NaCl, pH 7.2). Individual samples were then ultracentrifuged at 115,000g for 30 minutes at 4 °C. The buffer-expanded whey phase was (i) pipetted from the heavy pelleted precipitate and lighter lipid layer, (ii) placed in a clean tube, and (iii) immediately frozen to −90 °C. Diluted control mouse whey was identically prepared.

Antigen Assay Using Metal-dependent 7D7B10 Monoclonal Antibody

Each whey sample was diluted to 0.5 OD_{280} using TBS, which corresponded to a concentration of approximately 0.5 mg total protein per mL whey. Reference

dilutions of plasma-derived hPC were made (over the range of 0.003–2.0 µg hPC/mL whey) using control mouse whey prepared in TBS. Microtiter plate wells (96 wells; Dynatech, Alexandria, Virginia) were coated with 7D7B10 monoclonal antibody (Mab) by overnight incubation at 4 °C with 100 µL per well of 2 mg Mab/mL (0.1 M $NaHCO_3$, 25 mM EDTA, pH 9.3). The wells were then washed four times with TBS-TWEEN-EDTA (TBS, 0.05% Tween-80, 25 mM EDTA, pH 7.2). Whey samples consisting of 50 µL of either hPC standard or unknown were applied in triplicate to wells that contained 50 µL of TBS-PEG-EDTA buffer (TBS, 1 mg/mL 25K polyethylene glycol, 25 mM EDTA, pH 7.2). These were then incubated for 3 h at room temperature and washed four times with TBS-PEG-EDTA. The bound hPC or rhPC was then detected by sandwich enzyme-linked immunosorbent assay (ELISA). A 100-µL aliquot of a 1:1000 dilution of anti-hPC polyclonal antibody (American Bioproducts, Parsippany, New Jersey) prepared in TBS-PEG-EDTA was incubated in each well for 3 h at room temperature. The microtiter plates were then washed four times with TBS-PEG-EDTA and similarly incubated with 100 µL of a 1:1000 dilution of rabbit anti-mouse IgG–horseradish peroxidase conjugate (Sigma Chemicals, St. Louis, Missouri). The microtiter plates were again washed four times with TBS-PEG-EDTA and developed by addition of 100 µL of *ortho*-phenylene diamine (2.56 mg/mL) in OPD buffer (0.1 M citrate-phosphate, pH 5.0; Abbott Incorporated, Chicago, Illinois). The reaction was stopped after 4 minutes with 100 µL of 3 N H_2SO_4 per well.[36] The absorbance (OD_{490} nm) of the reaction product was read within 30 minutes of when the reaction was stopped.

Immunopurification

Immunoaffinity isolations of rhPC from whey and of hPC from Cohn IV-1 paste were done using the conformation-specific, metal-dependent monoclonal antibody (Mab) 7D7B10,[1,24,37] which was immobilized on Affiprep-10 (Bio-Rad Laboratories, Richmond, California) at 1–2 mg Mab/mL gel.[24] Expanded mouse whey, prepared in the manner described above, was thawed at 4 °C and filtered on ice through semicrimped rapid filter paper (product 9-795, Fisher Scientific, Pittsburgh, Pennsylvania). The total protein concentration was adjusted to less than 10 mg/mL with TBS-EDTA (TBS adjusted to a final concentration of 25 mM EDTA). The diluted whey was loaded batchwise onto the 7D7B10/Affiprep-10 immunosorbent for 3–4 hours at 4 °C. The gel was centrifuged at 3000 g for 5 minutes and loaded onto a 1 cm × 10 cm column (Pharmacia, Piscataway, New Jersey). The column was washed with TBS-EDTA at a flow rate of 30 mL/hour. The immunosorbed rhPC was eluted with TBS plus 25 mM $CaCl_2$. The column was then stripped with a step change to 4 M NaCl, followed by a step change to 2 M NaSCN solution, and then equilibrated with TBS-EDTA. The 25 mM $CaCl_2$, CaSCN, and NaCl eluates were dialyzed against deionized water at 4 °C for 12 h and then were lyophilized at 0.1 torr.

SDS-PAGE

Samples of immunopurified rhPC were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 0.1% SDS/10% acrylamide) under

either reduced or nonreduced conditions according to Laemmli[38] and were stained (0.125% Coomassie blue G-250, 50% methanol, 10% acetic acid). Identical SDS-PAGE gels were prepared and a Western immunoblot was performed according to the method of Towbin *et al.*[39]

Activated Partial Thromboplastin Time (APTT) Assay

The biological activity was measured by a delay in coagulation time obtained for rhPC and hPC samples prepared in hPC-deficient plasma (American Bioproducts, Parsippany, New Jersey). APTT reagent (Organon Teknika, Durham, North Carolina) included Protac (*Agkistrodon contortrix* venom, American Diagnostica, Greenwich, Connecticut) to specifically activate hPC or rhPC prior to initiating coagulation by adding $CaCl_2$.[40] Clotting times were recorded with an Electra 750A Coagulation Timer. A reference anticoagulation curve was prepared using a normal plasma reference pool (NPRP) whereupon the specific activities of Protein C in the samples were calculated assuming a theoretical activity of 1 unit per mL of NPRP and 4 µg/mL of hPC antigen in NPRP.

TABLE 1. Mouse Embryo Microinjection, Transfer, and Founder Animal Generation Data

Production Stage	Number
embryos injected	2336
embryos transferred	1808
number recipients	58
embryos per recipient	31
number pregnant	24
number pups alive	105
number pups transgenic	30
percent live pups transgenic	29
number transgenic pups female	16
number assayed for expression	6
number expressing	6

RESULTS

A total of 105 mice were born from recipients that had received embryos microinjected with the WAPPC-1 construct. Polymerase chain reaction analysis of tail DNA indicated that 30 mice contained the transgene (TABLE 1). FIGURE 2 demonstrates the facile nature and reliability of the detection of transgenic mice using the three-primer PCR method described earlier. Amplifications using transgenic mouse DNA as the template result in the formation of two amplification fragments. A predominant band appears at 402 bp, whereas a minor band occurs at 222 bp. These are the expected amplification fragment sizes for the transgene and the endogenous WAP, respectively. In contrast, DNA samples obtained from control

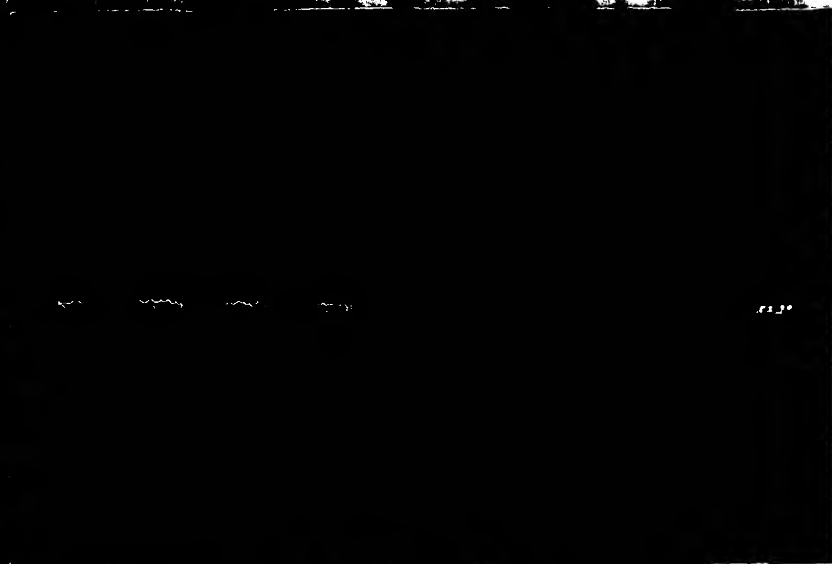

FIGURE 2. Example of detection of the WAPPC-1 transgene in mouse tail DNA by the polymerase chain reaction. Lanes 1, 5, and 9 are 4[illegible]-bp amplification products of plasmid DNA containing the WAPPC-1 construct. Lanes 2–4 show the 402-bp target fragment indicating the presence of the WAPPC-1 transgene in mouse tail DNA. Lanes 6–8 are amplifications of control mouse tail DNAs showing only a 222-bp fragment within the endogenous WAP gene. DNA templates consisted of either 10,000 plasmid copies for the standards (lanes 1, 5, and 9) or 10,000 genomes for the transgenic mice (lanes 2–4) and control mice (lanes 6–8).

mice containing only the single-copy endogenous WAP gene produce a single PCR product at 222 bp. Of 16 transgenic females generated, 6 were tested for expression and all 6 expressed hPC antigen in their milk at levels of [illegible]–3.0 μg/mL (TABLE 2). These animals appeared to exhibit a normal physiology. The hPC antigen levels were relatively stable with respect to the day of lactation, but the highest concentrations were observed for days 8–12.

Collection of milk three times per lactation using oxytocin yielded an average of 1 mL of milk per mouse. All milk samples from founder animals and their transgenic

TABLE 2. Expression Levels of Six Transgenic Founder Female Mice as Detected by Sandwich ELISA Using 7D7B10 Monoclonal Antibody Mass Capture[a]

	Expression of hPC Antigen (μg/mL)			
	Day of Lactation			
Mouse ID	5–6	8–9	11–12	13–15
R3	1.90	2.88	3.[illegible]	—
R12	—	2.98	2.[illegible]	2.40
Y51	—	2.80	1.3[illegible]	1.79
Y52	—	1.52	—	0.95
Y57	1.[illegible]7	—	0.9[illegible]	—
Y68	—	1.05	—	—

[a] A dash indicates "not analyzed".

TABLE 3. Immunopurified Recombinant Protein C from the Transgenic Mouse Whey Pool, Recovery Data from Immunoaffinity Chromatography, Anticoagulant Activity, and Comparison to Anticoagulant Activity of Human Plasma-derived Protein C[a]

Material Assayed	hPC Antigen Total (μg)	hPC Antigen % Yield	% Theoretical Anticoagulant Activity[b]
whole milk pool[c]	30	—	—
whey fraction	30	100	57 ± 10
immunopurified product	14	47	74 ± 2
plasma reference[d]	—	—	84 ± 14

[a] A total of 40 mice (founder mice and their transgenic offspring) were milked and 30 mL of milk was pooled and used for the immunopurification of rhPC.

[b] Percentage of theoretical specific activity calculated on a per mg basis of hPC antigen as detected by ELISA.

[c] The milk samples of transgenic founder animals and their offspring were combined to form a single pool.

[d] Theoretical APTT specific activity based upon 1 unit/mL for a normal human plasma pool at 4 μg hPC antigen/mL plasma. All samples were preactivated with venom of *Agkistrodon contortrix*.[40]

offspring were pooled and subjected to immunopurification using the 7D7B10 metal-dependent, conformation-specific monoclonal antibody. No hPC antigen was found to be specifically eluted by Ca^{2+}, NaCl or CaSCN from purifications using the control mouse whey pool (data not shown). Thus, no evidence of cross-reactivity between endogenous mouse milk proteins and the anti-hPC 7D7B10 Mab was seen during immunopurification. An overall hPC antigen yield of 46% was obtained for the Ca^{2+}-specific immunopurification from the transgenic mouse whey pool (TABLE 3).

The immunopurified material was evaluated by SDS-PAGE (FIGURE 3) and

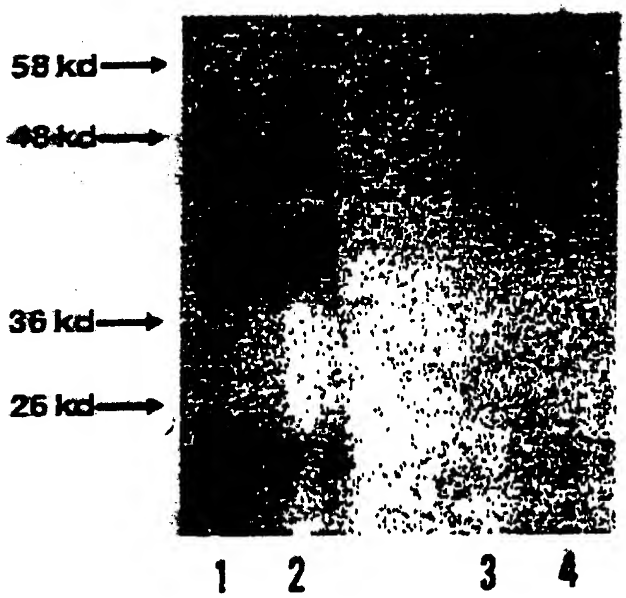

FIGURE 3. Sodium dodecylsulfate (0.1%) polyacrylamide gel (10%) electrophoresis of rhPC that was immunopurified from the transgenic mouse whey pool. Lanes 1 and 4 contain 5 μg of rhPC reference, reduced and nonreduced, respectively. Lanes 2 and 3 contain 5 μg of hPC reference, reduced and nonreduced, respectively.

compared to identical Western analysis (FIGURES 4a and 4b). Both the nonreduced Coomassie blue–stained SDS-PAGE and the Western immunoblot of rhPC and hPC showed the presence of multimeric bands of approximately 58,000 M_r. Protein C antigen assignment was made for the bands appearing on the reduced SDS-PAGE stained with Coomassie blue by comparison with the equivalent Western analysis.

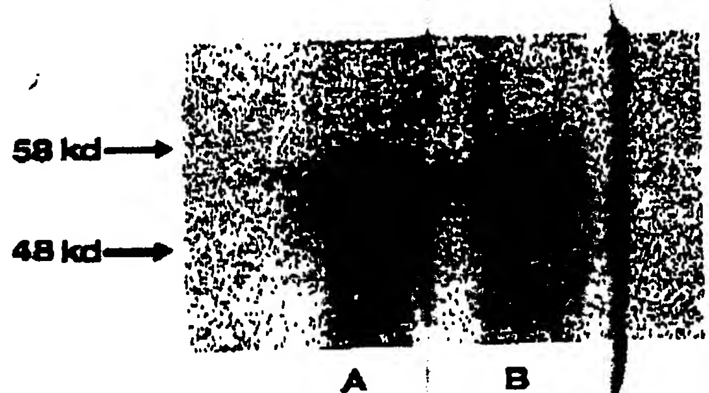

FIGURE 4a. Western immunoblot of nonreduced rhPC that was immunopurified from the transgenic mouse whey pool. Lanes A and B contain 250 ng of reference hPC and rhPC, respectively.

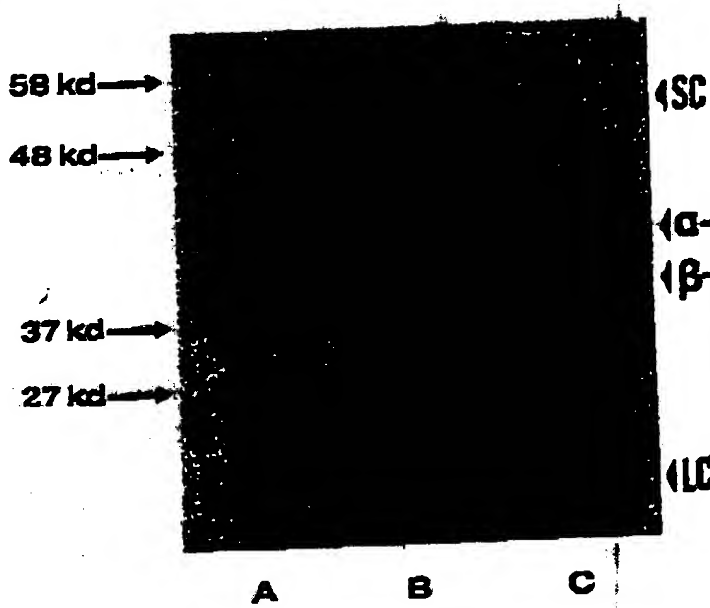

FIGURE 4b. Western immunoblot of reduced rhPC that was immunopurified from the transgenic mouse whey pool. Lanes A and C contain 250 ng of reference hPC. Lane B contains 250 ng of rhPC. The relative locations of the single chain (SC), the α and β heavy chains (HC), and the light chain (LC) are indicated on the right side.

Hence, both immunopurified hPC and rhPC products were determined to be greater than 95% pure by densitometry of the stained SDS-PAGE. The apparent heavy chain forms of rhPC occurring at approximately 40,000 M_r on reduced Western blots were slightly faster than the hPC in electrophoretic mobility. The rhPC heavy chain forms were similar in relative amounts to those of hPC as determined by densitome-

try of the reduced and stained SDS-PAGE (densitometry data not shown). The slightly faster mobilities for rhPC heavy chains indicated that these chains were approximately 1–2 kDa less in M_r than the heavy chains of hPC. A heavy chain form intermediate to apparent alpha and beta heavy chain species was visible in Western analysis of rhPC and hPC. This intermediate band was more pronounced and better resolved for the apparent beta heavy chain of rhPC than for the beta heavy chain of hPC. A ratio of 70% alpha to 30% beta-plus (intermediate plus beta form) of the total heavy chains (with less than 1% gamma form of the heavy chain) was observed for both rhPC and hPC on stained SDS-PAGE (densitometry data not shown). The single-chain form was present in less than 17% of the total stained protein for both immunopurified rhPC and hPC (densitometry data not shown), although single-chain rhPC appeared to be more immunoreactive than two-chain hPC on the reduced Western blot (FIGURE 4b). The light chain of rhPC, appearing at approximately 21,000 M_r, migrated slightly faster than that of hPC, with the apparent M_r difference being less than 1–2 kDa.

The percentage of theoretical specific anticoagulant activity of the rhPC (74% ± 2%) was essentially equivalent to that of the hPC reference material (84% ± 14%) (TABLE 3) as measured by the APTT assay.

DISCUSSION

WAP regulatory elements have been demonstrated previously to direct the expression of heterologous genes in the mammary tissue of mice[10] and pigs.[13] Here, a WAP genomic clone was used to drive the expression of the cDNA for human Protein C into the milk of transgenic mice. These studies here were important for demonstrating the capability of the murine mammary gland to produce a biologically active VKD protein throughout lactation. The biologically active rhPC was produced by transgenic mice at levels of 0.5–3 μg/mL milk with milk letdowns approximately every hour. Although the amino acid sequence of the rhPC has not been determined, the high specific anticoagulant activity of the rhPC is strong evidence for the presence of a functional serine protease catalytic site and properly carboxylated glutamic acid residues. These gla residues are indicative of a functional membrane-binding domain.[20] Our results strongly contrast the very low specific activity reported for the VKD-factor IX expressed in the milk of transgenic sheep at only 25 ng/mL.[17] Furthermore, the extent of proteolytic removal of the dipeptide appears to be similar to that in human plasma because the ratio of the single-chain to the two-chain form for rhPC was within the published range of 5–30% for hPC.[30] The multiple forms of heavy chain hPC species have been attributed previously to different sites of glycosylation.[2,31] The ratio of apparent triglycosylated forms (alpha-form heavy chain) to diglycosylated forms (beta-form heavy chain) of dimeric rhPC appears similar to that of the hPC and, thus, the glycosylation selectivity of the murine mammary gland appears comparable to that of human liver. The very slight differences in M_r between the heavy chain forms of hPC and th[illegible] may be due to slight differences in branched carbohydrate structure. The similarity in light chain mobility observed for both rhPC and hPC is consistent with a comparable incidence of N-linked glycosylation that occurs at ASN97.[29,31] However, the extent of propeptide

cleavage has not yet been determined for rhPC. Direct [illegible]ical analyses of the carbohydrate content and amino acid sequence of rhPC [illegible]urrently being performed.

This study of the expression of rhPC in the milk of tra[illegible]e mice demonstrates that the murine mammary tissue can perform a variety of [illegible]ex posttranslational modifications that had not been shown in previous studie[illegible]ddition, the present study provides valuable information about the functional[illegible] the WAPPC-1 construct. Both the transgene and its ability to express rh[illegible]C [illegible]pear to have been transmitted to offspring in a Mendelian fashion (data not [illegible]n). We are currently studying the differences in the WAPPC-1 gene structur[illegible]ween generations of mice. We are also awaiting results from the lactation of tr[illegible]nic swine containing the WAPPC-1 transgene. Upon completion of the latter st[illegible] more will be known about the apparent conservation of WAP regulation that [illegible] between mice[10] and pigs.[13] Most importantly, knowledge will be gained about [illegible]asibility of using the mammary gland of animals as a bioreactor for the product[illegible] complex therapeutic proteins.

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Exhibit C

US005252285A

United States Patent [19]

Lock

[11] **Patent Number: 5,252,285**

[45] **Date of Patent: Oct. 12, 1993**

[54] **PROCESS FOR MAKING SILK FIBROIN FIBERS**

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[21] Appl. No.: **827,141**

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[52] **U.S. Cl.** **264/202**; 264/204; 264/211.11; 264/211.16

[58] **Field of Search** 264/202, 204, 211.11, 264/211.16

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[57] **ABSTRACT**

The present invention relates to a process for spinning silk fibers. The process involves dissolving silk fibroin in an aqueous salt solution, removing the salt from the solution, followed by removal of the water to form a regenerated silk material. The silk material is then dissolved in hexafluoroisopropanol to form a fiber-spinnable solution.

8 Claims, No Drawings

PROCESS FOR MAKING SILK FIBROIN FIBERS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a process for spinning silk fibers. More specifically, the invention involves forming silk fibers by dissolving silk fibroin in an aqueous salt solution, removing the salt from the solution, followed by removal of the water, and redissolution of the resulting regenerated silk in hexafluoroisopropanol (HFIP) to produce a fiber-spinnable solution. The solution can be spun and drawn to produce high-quality fibers with near-native silk properties having greater mechanical strength.

2. Description of the Related Art

Silk fibroin (silkworm silk) is a naturally occurring polypeptide which occurs in fibrous form having high strength and a soft hand. The nature of silk fibroin makes it suitable for a wide range of uses including textile applications and in suture materials. Silk has been used as a suture material since ancient times. Because silkworms produce filaments in only one size (ca. 1 denier), twisted or braided yarns must be used when loads exceed a few grams. Unfortunately, the interstices of a multifilament yarn can be a route for infection. Thus, it would be desirable to be able to produce silk fibers in deniers other than those found in nature which would be suitable for such applications as monofilament sutures.

Fibroin is known to be soluble in certain high ionic strength aqueous salt solutions, for example, aqueous lithium thiocyanate (LiSCN), sodium thiocyanate (NaSCN), calcium thiocyanate ($Ca(SCN)_2$), magnesium thiocyanate ($Mg(SCN)_2$), calcium chloride ($CaCl_2$), lithium bromide (LiBr), zinc chloride ($ZnCl_2$), magnesium chloride ($MgCl_2$), and copper salts, such as copper nitrate ($Cu(NO_3)_2$), copper ethylene diamine ($Cu(NH_2CH_2CH_2NH_2)_2(OH)_2$), and $Cu(NH_3)_4(OH)_2$. It has long been known that the salts can be dialyzed out of such aqueous salt/fibroin solutions to produce aqueous solutions of fibroin which are similar in some ways to the liquid contents of a silkworm's silk gland. Fibers have been spun from aqueous fibroin solutions of this type, but more commonly, the solutions have been used to cast films for structure studies.

For example, Bhat and Ahirrao, *Journal of Polymer Science* Vol. 21, pp. 1273–1280 (1983) describe the dissolution of silk fibers in 70% lithium thiocyanate solution and regenerating the dissolved silk by casting films from the solution after dialyzing. They found that the cast films were amorphous and could o be transformed to a beta-sheet form using a variety of methods.

Those skilled in the art have attempted to find suitable solvents for preparing silk fibroin solutions which may be subsequently spun into fibers.

For example, Otoi et al., Japanese Kokoku Patent No. SHO 57[1982]-4723 describe a method for preparing a silk spinning solution involving dissolution of fibroin in an aqueous solution of copper-ethylenediamine, copper hydroxide-ammonia, copper hydroxide-alkali-glycerin, lithium bromide, sodium thiocyanate, or nitrates or thiocyanates of zinc, calcium, or magnesium. The solution is then dialyzed using a multilayered structure and used to fabricate fibers or films.

Bley, U.S. Pat. No. RE 22,650, discloses preparing fiber-spinnable polypeptide solutions containing a protein selected from the group consisting of silk fibroin, casein, gelatin, wool, and alginic acid in a solvent selected from quaternary benzyl-substituted ammonium bases.

U.S. Pat. No. 4,171,505 (Lock) describes a process for spinning polypeptide fibers including preparing fibers from a spinnable solution of silk fibroin in a solvent mixture of formic acid and lithium chloride.

Although it has been possible to produce silk fibroin fibers from such spinning solutions as described above, these solvents tend to be harsh and may degrade the fibroin. Dichloroacetic acid and trifluoroacetic acid are especially harsh and subject the polymer to a measurable degree of degradation. Fibers prepared from such solutions tend to be deficient in certain physical properties, such as mechanical strength.

Thus, a desirable solvent for preparing silk fibroin solutions is hexafluoroisopropanol (HFIP), because there is no detectable degradation of the fibroin in this solvent. However, in the past, it has not been possible to prepare silk fibers from HFIP solutions, since natural silk fibroin is not soluble in this solvent. Now, in accordance with this invention, a method for preparing silk fibroin fibers from silk fibroin/HFIP solutions has been discovered.

SUMMARY OF THE INVENTION

The present invention relates to a process for producing silk fibroin fibers. The process involves forming a silk fibroin solution of fibroin in an aqueous salt solution and removing the salt and water from the solution to form a fibroin material, such as a film. A fiber-spinnable solution comprising about 5 to 25% by weight of the silk fibroin material in hexafluoroisopropanol is then formed and extruded through a spinneret orifice to form a silk fiber.

Preferably, the aqueous salt solution includes a salt compound selected from the group consisting of lithium thiocyanate, copper (ethylene diamine) hydroxide, and zinc chloride. The salt may be removed by dialysis. The solution may be spun into fibers by wet-spinning, dry-jet wet spinning, or dry-spinning techniques. The invention also includes fiber-spinnable solutions and fibers produced from this process.

DETAILED DESCRIPTION OF THE INVENTION

In native fiber-form, silk fibroin is not soluble in hexafluoroisopropanol (HFIP), thus fibers cannot be spun from these solutions. It is believed that the density of hydrogen bonding between highly oriented polymer molecules in the beta-sheet structure of the fiber provides more cohesion than the solvent, HFIP, can overcome.

The present invention provides a method for producing fibers from natural silk fibroin / HFIP solutions. The silk is "respun" into fibers under conditions which do not result in polymer degradation, loss of molecular weight, and consequent loss of fiber physical properties. The silk fibers of this invention are chemically similar to native silkworm silk but have filament deniers, filament cross sections, etc., not o found in nature.

The process of the current invention involves the steps of 1) dissolution of silk fibroin which is insoluble in HFIP in an aqueous salt solution, 2) removal of the salt, 3) removal of the water to yield fibroin which is now soluble in HFIP, and 4) dissolution in HFIP, followed

by spinning of the solution through a spinneret orifice to obtain silk fibers.

It is preferable to purify the silk fibroin prior to dissolving in the aqueous salt solution. Methods for purification of fibroin are well known in the art.

The aqueous salt solution may be any of those known in the art for dissolving silk fibroin. The preferred salts are lithium thiocyanate, copper-(ethylene diamine) hydroxide and zinc chloride. Salts which may also be used include the nitrate, chloride and thiocyanate salts of calcium, magnesium, and zinc, and copper salts such as $Cu(NH_3)_4(OH)_2$. The concentration of salt in the solution must be sufficient to dissolve the fibroin. Concentrations of salt in the range of about 40 to 80 weight percent (wt. %) are preferred.

It is preferable to dissolve the fibroin at room temperature, however elevated temperatures may be used, up to about 80° C., in order to increase the rate of dissolution. Heating should not be conducted at a temperature at which the fibroin may be degraded. Fibroin solutions in aqueous lithium thiocyanate are stable on standing several days. Preferably, the concentration of silk fibroin in the aqueous salt solution is in the range of about 5 to 40 weight percent. If the concentration of fibroin is less than about 5 weight percent, the solution is difficult to handle, since the salt must be dialyzed and high amounts of water removed. If the concentration of fibroin is greater than about 40 weight percent, the solution is difficult to handle because of its high viscosity.

Once the fibroin is dissolved in the salt solution, the salt is removed using methods known in the art. Preferably, this removal is done by dialysis of the solution.

The fibroin is isolated from the desalted or dialyzed solution by removal of the water. This may be done using a number of methods known in the art. A convenient means is by casting of films and removal of the water by evaporation. The solution may also be lyophilized or spray dried, or the solvent removed in a rotary evaporator.

Surprisingly, the resulting regenerated fibroin material is readily soluble in HFIP, whereas it was not soluble prior to the dissolution process described above. It is believed that the fibroin molecules in the films cast from the aqueous solutions of this invention are typically not in highly oriented beta-sheets and are therefore not extensively involved in high-density hydrogen bonding. This reduced crystalline structure of the fibroin allows it to be re-dissolved in HFIP solution from which fibers may be spun. It has been found that films as old as six months can be readily dissolved in HFIP.

Preferably, the HFIP solution is prepared by dissolving the regenerated fibroin in the HFIP solvent at room temperature. The solutions may be safely heated at temperatures up to about 30° C. for several hours if desired. Concentrations of the fibroin should be such as to yield fiber-spinnable solutions. Concentrations of about 5 to 25 weight percent have been found to be useful, with concentrations of 10 to 20 weight percent being preferred.

The spinnable solution may then be spun into fibers using elements of processes known in the art. These processes include, for example, wet spinning, dry-jet wet spinning, and dry spinning. Wet spinning is preferred as it is the simpler of these processes.

In a wet spinning process, the spinning solution is extruded directly into a coagulating bath. The coagulant may be any fluid wherein the hexafluoroisopropanol is soluble, but wherein the silk is insoluble. Examples of suitable coagulating fluids include water, methanol, ethanol, isopropyl alcohol, and acetone. Methanol has been found to be the preferred coagulating fluid. The fibers may be cold drawn while still wet with coagulating fluid. Preferably, the fibers are dried under tension in order to prevent shrinkage and to obtain improved tensile properties.

In a dry-jet wet spinning process, the spinning solution is attenuated and stretched in an inert, noncoagulating fluid, e.g., air, before entering the coagulating bath. Suitable coagulating fluids are the same as those used in a wet spinning process.

In a dry spinning process, the spinning solution is not spun into a coagulating bath. Rather, the fibers are formed by evaporating the solvent into an inert gas which may be heated.

Testing Methods

Physical properties such as tenacity, elongation, and initial modulus were measured using methods and instruments which conformed to ASTM Standard D 2101-82, except that the test specimen length was one inch. Five breaks per sample were made for each test.

The following examples further describe the invention but should not be construed as limiting the scope of the invention. In these examples, parts and percentages are by weights, unless otherwise indicated.

EXAMPLE

Preparation of Degummed Silk Fibroin

Purified silk fibroin may be prepared from raw reeled silk yarn or from cocoons which have been cut open, had the pupae removed, and been picked clean of foreign vegetative matter.

Purified silk fibroin was prepared from raw reeled silk yarn by boiling a 160 g hank at reflux in 3.3 liters of deionized water with 1.75 g sodium carbonate and 10.5 g powdered "Ivory" soap for 1.5 hours. After boiling, the silk was removed from the water, wrung out, and rinsed twice in 3 liter portions of hot deionized water. The rinsed silk was then boiled again at reflux in 3.3 liters of deionized water with 0.66 g sodium carbonate for 1 hour, removed, wrung out, and rinsed twice in 3 liter portions of hot deionized water. Finally, the silk was wrung out thoroughly, soaked ½ hour in each of two 1 liter portions of methanol, wrung thoroughly, and allowed to dry in the room temperature air flow of a laboratory fume hood. The product was 124.5 g purified silk fibroin, still in fiber form.

Physical testing of the silk fibroin filaments showed them to be 0.66–1.04 dtex (0.59–0.94 denier), 0.86 dtex average (0.77 denier) with tenacities of 3.21–4.23 dN/tex (3.64–4.79 gpd (grams per denier)), 3.84 dN/tex average (4.35 gpd), elongations of 11.5–31.2% (20.5 % average), and initial moduli of 59.5–77.5 dN/tex (67.4–87.8 gpd), 70.0 dN/tex average (78.1 gpd).

Preparation of Lithium Thiocyanate/Fibroin Solution.

A stock solution was prepared by dissolving 100 g lithium thiocyanate hydrate ($LiSCN \times H_2O$, Aldrich, ca. 60 wt. % LiSCN / 40 wt. % H_2O) in 43 g deionized water. The solution was filtered to remove insoluble contaminants.

A solution of 20% silk fibroin in aqueous lithium thiocyanate was prepared by mixing 10.29 g purified silk fibroin, above, with 41.02 g of the LiSCN stock solution in a small plastic packet made by heat-sealing

sheets of 5 mil polyethylene film. The mixture initially became thick and foamy as the silk fiber disintegrated and dissolved. However, on standing three days with intermittent vigorous mixing, the mixture became a clear, viscous, pale amber solution.

Dialysis of Lithium Thiocyanate/Fibroin Solution.

An aqueous solution of silk fibroin was prepared by dialyzing the lithium thiocyanate solution above.

The solution of silk fibroin in aqueous lithium thiocyanate was filtered through a stack of stainless steel screens of 50, 325, 325, and 50 mesh and transferred into two (ca. 25 cm) lengths of 32 mm flat width "Spectrapor" viscose process cellulose dialysis tubing with 12-14,000 molecular weight cutoff. Tubing ends were sealed with clamps. Dialysis was carried out by placing the cellulose membrane tubes containing the silk/LiSCN solution into a shallow pan of deionized water and allowing a trickle of deionized water to flow into the pan and overflow into a drain. After 20 hours, the dialysis was considered complete. The resulting solution of silk fibroin in water was nearly clear and quite free-flowing but had very unusual surface tension properties, like a thin egg white. It was slightly sticky to the touch, and readily picked up small, quite stable air bubbles.

Casting of Fibroin Film

The aqueous solution of silk fibroin prepared by dialysis above was spread on flat polyethylene sheets using a 20 mil doctor knife and allowed to stand in room air to dry overnight. This produced 9.19 g of thin, transparent, slightly sticky, cellophane-like silk fibroin film.

Preparation of Fibroin HFIP Solution

A solution containing 14.9% silk fibroin film in the solvent hexafluoroisopropanol (HFIP) was prepared by adding 5.70 g HFIP to 1.00 g of film in a heat-sealed polyethylene packet, mixing thoroughly, and allowing the mixture to stand for 8 days with intermittent vigorous mixing. The solution was thick, clear, and a light yellowish pink in color.

Wet Spinning of Silk Fibers from HFIP Solution

The solution of silk fibroin in HFIP was transferred to a syringe fitted with a stainless steel screen pack consisting, in order, of 50, 325, 325, and 50 mesh screens. The syringe was capped and centrifuged to disengage air bubbles trapped in the solution. A syringe pump was then used to force the solution through the screen pack and out of the syringe through a 5 mil (0.013 cm) diameter by 10 mil (0.025 cm) length orifice in a stainless steel spinneret directly into a container of methanol at room temperature. The syringe pump was set to deliver the solution at a rate of 0.0136 ml/min. The filament which formed as the solution was extruded into methanol was allowed to fall freely and to coil on itself at the bottom of the container.

The coiled filament was allowed to stand in methanol overnight. Then, while still wet with methanol, the filament was drawn to 4× its length. The ends of the drawn fiber were fixed in place to prevent shrinkage during drying in room air.

Physical testing of samples of the dry fiber showed them to be 24.4-29.4 dtex (22.0-26.5 denier), 27.4 dtex average (24.7 d) with tenacities of 3.83-4.81 dN/tex (4.34-5.45 gpd), 4.20 dN/tex average (4.76 gpd), elongations of 8.2-9.3% (8.9% average), and initial moduli of 78.4-126.1 dN/tex (88.8-142.8 gpd), 101.1 dN/tex average (114.5 gpd). The above figures indicate that the tenacity and modulus of the "respun" silk fiber exceeded the tenacity and modulus of the native silk fiber.

COMPARATIVE EXAMPLE A

This example demonstrates the insolubility of natural silk fiber in hexafluoroisopropanol (HFIP).

An attempt was made to dissolve purified silk fibroin fiber directly in HFIP. 0.763 g of purified fiber was mixed with 4.35 g of HFIP in a heat-sealed polyethylene packet. The solvent had essentially no effect on the fiber beyond a slight swelling, even after 1 month. Gentle heating (to 40° C.) also produced no apparent changes.

I claim:

1. A process for producing silk fibroin fibers, comprising the steps of:
   a) forming a silk fibroin solution comprising silk fibroin in an aqueous salt solution;
   b) removing the salt and water from the fibroin solution to form a silk fibroin material;
   c) forming a fiber-spinnable solution comprising about 5 to 25% by weight of the silk fibroin material in hexafluoroisopropanol; and
   d) extruding the fiber-spinnable solution through a spinneret to form silk fibroin fibers.

2. The process of claim 1, wherein the aqueous salt solution comprises a salt compound selected from the group consisting of lithium thiocyanate, copper(ethylene diamine) hydroxide, and zinc chloride.

3. The process of claim 2, wherein the salt compound is lithium thiocyanate.

4. The process of claim 1, wherein the salt is removed by dialysis, and the water is evaporated to form a silk fibroin film.

5. The process of claim 1, wherein the solution is extruded directly into a liquid coagulating medium to remove the hexafluoroisopropanol.

6. The process of claim 1, wherein the solution is extruded into an inert, non-coagulating fluid, and then into a liquid coagulating medium to remove the hexafluoroisopropanol.

7. The process of claim 5 or 6, wherein the liquid coagulating medium is methanol.

8. The process of claim 1, wherein the solution is extruded into an inert gas to remove the hexafluoroisopropanol.

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